


# PATENT COOPERATION TREATY

## PCT

### INTERNATIONAL PRELIMINARY REPORT ON PATENTABILITY

(Chapter II of the Patent Cooperation Treaty)

(PCT Article 36 and Rule 70)

|  |  |   |  |  |
|--|--|---|--|--|
| Applicant's or agent's file reference<br>SCB 866 PCT   |  | <b>FOR FURTHER ACTION</b>   |  | See Form PCT/IPEA/416                        |
| International application No.<br>PCT/EP2004/008245   |  | International filing date (day/month/year)<br>23.07.2004                    |  | Priority date (day/month/year)<br>29.07.2003 |
| International Patent Classification (IPC) or national classification and IPC<br>A61K38/18, A61P7/00  |  |   |  |  |
| Applicant<br>DOMPE' S.P.A. et al.  |  |   |  |  |
| <p>1. This report is the international preliminary examination report, established by this International Preliminary Examining Authority under Article 35 and transmitted to the applicant according to Article 36.</p> <p>2. This REPORT consists of a total of    sheets, including this cover sheet.</p> <p>3. This report is also accompanied by ANNEXES, comprising:</p> <p>a. <input checked="" type="checkbox"/> sent to the applicant and to the International Bureau a total of 18 sheets, as follows:</p> <p style="margin-left: 20px;"><input checked="" type="checkbox"/> sheets of the description, claims and/or drawings which have been amended and are the basis of this report and/or sheets containing rectifications authorized by this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions).</p> <p style="margin-left: 20px;"><input type="checkbox"/> sheets which supersede earlier sheets, but which this Authority considers contain an amendment that goes beyond the disclosure in the international application as filed, as indicated in item 4 of Box No. I and the Supplemental Box.</p> <p>b. <input type="checkbox"/> (sent to the International Bureau only) a total of (indicate type and number of electronic carrier(s)) , containing a sequence listing and/or tables related thereto, in computer readable form only, as indicated in the Supplemental Box Relating to Sequence Listing (see Section 802 of the Administrative Instructions).</p> |  |   |  |  |
| <p>4. This report contains indications relating to the following items:</p> <p><input checked="" type="checkbox"/> Box No. I    Basis of the opinion</p> <p><input type="checkbox"/> Box No. II    Priority</p> <p><input type="checkbox"/> Box No. III    Non-establishment of opinion with regard to novelty, inventive step and industrial applicability</p> <p><input type="checkbox"/> Box No. IV    Lack of unity of invention</p> <p><input checked="" type="checkbox"/> Box No. V    Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement</p> <p><input type="checkbox"/> Box No. VI    Certain documents cited</p> <p><input type="checkbox"/> Box No. VII    Certain defects in the international application</p> <p><input type="checkbox"/> Box No. VIII    Certain observations on the international application</p>   |  |   |  |  |
| Date of submission of the demand<br><br>04.05.2005   |  | Date of completion of this report<br><br>07.07.2005                         |  |  |
| Name and mailing address of the international preliminary examining authority:<br><br> European Patent Office<br>D-80298 Munich<br>Tel. +49 89 2399 - 0 Tx: 523656 epmu d<br>Fax: +49 89 2399 - 4465  |  | Authorized Officer<br><br>Bochelen, D<br><br>Telephone No. +49 89 2399-8150 |  |  |



**INTERNATIONAL PRELIMINARY REPORT  
ON PATENTABILITY**

International application No.  
PCT/EP2004/008245

**Box No. I Basis of the report**

1. With regard to the **language**, this report is based on the international application in the language in which it was filed, unless otherwise indicated under this item.
  - ☐ This report is based on translations from the original language into the following language , which is the language of a translation furnished for the purposes of:
    - ☐ international search (under Rules 12.3 and 23.1(b))
    - ☐ publication of the international application (under Rule 12.4)
    - ☐ international preliminary examination (under Rules 55.2 and/or 55.3)
2. With regard to the **elements\*** of the international application, this report is based on *(replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report):*

**Description, Pages**

|                             |                                  |
|-----------------------------|----------------------------------|
| 1-3, 5, 8-11, 18, 19, 29-35 | as originally filed              |
| 4, 6-7, 12-17, 20-28        | filed with telefax on 04.05.2005 |

**Claims, Numbers**

1-10 as originally filed

- ☐ a sequence listing and/or any related table(s) - see Supplemental Box Relating to Sequence Listing

3. ☐ The amendments have resulted in the cancellation of:
  - ☐ the description, pages
  - ☐ the claims, Nos.
  - ☐ the drawings, sheets/figs
  - ☐ the sequence listing (*specify*):
  - ☐ any table(s) related to sequence listing (*specify*):
4. ☐ This report has been established as if (some of) the amendments annexed to this report and listed below had not been made, since they have been considered to go beyond the disclosure as filed, as indicated in the Supplemental Box (Rule 70.2(c)).
  - ☐ the description, pages
  - ☐ the claims, Nos.
  - ☐ the drawings, sheets/figs
  - ☐ the sequence listing (*specify*):
  - ☐ any table(s) related to sequence listing (*specify*):

\* If item 4 applies, some or all of these sheets may be marked "superseded."

**INTERNATIONAL PRELIMINARY REPORT  
ON PATENTABILITY**

International application No.  
PCT/EP2004/008245

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**Box No. V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

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**1. Statement**

|                               |             |      |
|-------------------------------|-------------|------|
| Novelty (N)                   | Yes: Claims | 1-10 |
|                               | No: Claims  |      |
| Inventive step (IS)           | Yes: Claims | 1-10 |
|                               | No: Claims  |      |
| Industrial applicability (IA) | Yes: Claims | 1-10 |
|                               | No: Claims  |      |

**2. Citations and explanations (Rule 70.7):**

**see separate sheet**

**INTERNATIONAL PRELIMINARY  
REPORT ON PATENTABILITY  
(SEPARATE SHEET)**

**10/565903**  
**IAP20 Rec'd PCT/PTO 25 JAN 2006**  
International application No.

**PCT/EP2004/008245**

**Re Item V.**

**1. The following documents are referred to in this communication:**

- D1 : KADAR J G ET AL: "Technical and safety aspects of blood and marrow transplantation using G-CSF mobilized family donors" TRANSFUSION SCIENCE, PERGAMON PRESS, OXFORD, GB, vol. 17, no. 4, December 1996 (1996-12), pages 611-618, XP004568854 ISSN: 0955-3886**
- D2 : CARMELIET P ET AL: "SYNERGISM BETWEEN VASCULAR ENDOTHELIAL GROWTH FACTOR AND PLACENTAL GROWTH FACTOR CONTRIBUTES TO ANGIOGENESIS AND PLASMA EXTRAVASATION IN PATHOLOGICAL CONDITIONS" NATURE MEDICINE, NATURE PUBLISHING, CO, US, vol. 7, no. 5, May 2001 (2001-05), pages 575-583, XP001017902 ISSN: 1078-8956**
- D3 : HATTORI KOICHI ET AL: "Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment." NATURE MEDICINE. AUG 2002, vol. 8, no. 8, August 2002 (2002-08), pages 841-849, XP002307301 ISSN: 1078-8956**
- D4 : DE REVEL THIERRY ET AL: "Effects of granulocyte colony-stimulating factor and stem cell factor, alone and in combination, on the mobilization of peripheral blood cells that engraft lethally irradiated dogs" BLOOD, vol. 83, no. 12, 1994, pages 3795-3799, XP002307302 ISSN: 0006-4971**
- D5 : CARLO-STELLA CARMELO ET AL: "Defibrotide in combination with granulocyte colony-stimulating factor significantly enhances the mobilization of primitive and committed peripheral blood progenitor cells in mice" CANCER RESEARCH, vol. 62, no. 21, 1 November 2002 (2002-11-01), pages 6152-6157, XP002307303 ISSN: 0008-5472**

If not indicated otherwise the relevant passages are those mentioned in the search report.

Document D1 discloses the mobilisation of haematologic progenitor cells with G-CSF.

Document D2 discloses the recruitment of haematopoietic stem cells by VEGF and PlGF.

Document D3 discloses that PIGF is implicated in the mobilisation of bone marrow cells.

Document D4 discloses that stem cell factor and G-CSF mobilize peripheral blood haematopoietic precursors.

Document D5 discloses that Defibrotide and rhG-CSF mobilize peripheral blood progenitor cells.

**2. Novelty and inventive step (Art. 33(1) PCT):**

**2.1** The subject-matter of claims 1 and 6 is delimited from documents D1-D5 in that Placental Growth Factor and Granulocyte-Colony Stimulating Factor are combined. Claims 1 and 6 thus fulfill the requirements of Art. 33(1) (2) PCT.

**2.2** Documents D1 and D4-D5 disclose that G-CSF alone or in combination with stem cell factor or Defibrotide mobilizes blood progenitor cells. Documents D2-D3 disclose that PIGF mobilizes bone marrow cells and peripheral blood hemapoietic precursors. The combination of G-CSF and PIGF results in a synergistic effect with regard to the mobilization of blood stem cells (see examples 1-11). Consequently, it is considered that claims 1 and 6 fulfill the requirements of Art. 33(1) (3) PCT with regard to inventive step. Dependent claims 2-5 and 7-10 as well meet the requirements of the PCT in respect of inventive step.

IAP20 Rec'd PCT/PTO 25 JAN 2006

stem cell mobilization, we tested the mobilizing activity of PlGF in animal models allowing to simulate PBPC mobilization as occurring in a clinical situation. Normal BALB/c mice were injected intraperitoneally (IP) for 5 days with either control vehicle (PBS/MSA), rhG-CSF alone (10 µg/d), or a combination of rhG-CSF (10 µg/d) ~~with either~~ and recombinant murine (rm)PlGF (2.5 - 5 µg/d) ~~or recombinant human (rh)PlGF (5 - 10 µg/d)~~. Blood samples were collected 2 hours after the last injection of cytokines and the following parameters were evaluated: white blood cell (WBC) counts, frequency and absolute numbers of colony-forming cells (CFC), absolute numbers of long-term culture-initiating cells (LTC-IC).

The effects of rmPlGF are illustrated in Tables 1 - 4 below. It is evident that rmPlGF injected alone has no effect on the mobilization of WBC, CFC, and LTC-IC. A 5-day injection of rmPlGF (5 µg/d) combined with rhG-CSF significantly increases mobilization of CFC and LTC-IC, as compared to rhG-CSF alone.

~~Tables 5 - 8 summarize the mobilizing effects of rhPlGF. Again, rhPlGF has no effects on circulating WBC or hematopoietic progenitors when injected alone. In contrast, the combined injection of rhPlGF and rhG-CSF significantly increases mobilization of CFC and LTC-IC, as compared to rhG-CSF alone.~~

~~We also tested the mobilizing effects of a 12-day treatment with rhPlGF (10 µg/d) and rhG-CSF (10 µg/d). Mice receiving the 12-day treatment were analyzed on days 5, 8, 10, and 12 of therapy. As compared to rhG-CSF alone, the combined rhPlGF/rhG-CSF treatment significantly increased the frequency and the absolute number of blood CFC at each time point analyzed in our study (Tables 9 - 11).~~

In addition, the mobilizing activity of PlGF/G-CSF combinations was tested in a non-human primate model (Rhesus Monkeys). The results obtained

stem cell mobilization. The patient/subject response can be monitored during the treatment, e.g. by counting the circulating blood stem cells, and if necessary the dosages can be modified accordingly. In a preferred embodiment of the invention, recombinant hG-CSF and rhPlGF are used in  
5 form of injectable solutions supplying a daily amount of the active comprised from 1 to 150, preferably from 5 to 20  $\mu\text{g/kg}$  G-CSF and from 10 to 300, preferably from 20 to 150  $\mu\text{g/kg}$  PlGF.

The following examples further illustrate the invention.

**EXAMPLES 1-11 - mobilizing effects of PIGF/G-CSF combination**  
10 **in a mouse model**

**MATERIALS AND METHODS**

**Animals.** Six- to 8-week-old female BALB/c mice, with body weight of 20 to 25 g, were purchased from Charles River (Milano, Italy, EU). Experimental procedures performed on animals were carried out in accordance  
15 with the guidelines of the United Kingdom Coordinating Committee on Cancer Research (UK Coordinating Committee on Cancer Research. UKCCCR guidelines for the welfare of animals in experimental neoplasia. Br. J. Cancer., 58:109-113, 1998.). The mice were injected daily, intraperitoneally (IP), for 5 days with either control vehicle (PBS/MSA), rhG-CSF alone  
20 (10  $\mu\text{g/d}$ ), or a combination of rhG-CSF (10  $\mu\text{g/d}$ ) with either recombinant murine (rm)PlGF (2.5 - 5  $\mu\text{g/d}$ ) or recombinant human (rh)PlGF (5 - 10  $\mu\text{g/d}$ ). Each experiment was performed at least on three separate occasions, and three to four mice per group per time point were used.

**Cytokines.** Recombinant human granulocyte colony-stimulating factor  
25 (rhG-CSF, Neupogen®) was from Roche (Milan, Italy, EU); rmPlGF was purchased from R&D Systems Inc., Abingdon, United Kingdom); rhPlGF was provided from Geymonat SpA (Anagni, Italy, EU).

**Mobilization protocols.** The standard mobilization protocol included

treatment of BALB/c with rhG-CSF (10 µg/mouse, IP) once daily for 5 days. To evaluate the mobilizing effects of PlGF, rmPlGF (2.5 - 5 µg/mouse, IP) or rhPlGF (5 - 10 µg/mouse, IP) were administered once daily for 5 days either as a single agent or in combination with rhG-CSF. The mobilizing effects of rhPlGF were also tested by a 12 day treatment with rhPlGF (10 µg/mouse/day) and rhG-CSF (10 µg/mouse/day). Controls were injected with PBS/MSA.

**Mobilization parameters.** Mobilization was evaluated by white blood cell (WBC) counts, frequency and absolute numbers of colony-forming cells (CFC), absolute numbers of long-term culture-initiating cells (LTC-IC). Unless otherwise stated, animals were sacrificed two hours after the last treatment.

**Cell harvesting and separation.** PB was harvested from the orbital plexus into heparin-containing tubes. After white blood cell (WBC) counting, PB was diluted (1:4, v/v) with PBS and mononuclear cells (MNCs) were separated by centrifugation (280 g, 30 min, room temperature) on a Ficoll discontinuous density gradient. Cells were then washed twice in Iscove's modified Dulbecco's medium (IMDM, Seromed, Berlin, Germany, EU) supplemented with 10% fetal bovine serum (FBS, Stem Cell Technologies, Vancouver, Canada), 2 mM L-glutamine and antibiotics.

**WBC counts.** WBC counts were performed using heparin-anticoagulated blood and an automated counter (ADVIA 120, Bayer, Milano, Italy, EU).

**Colony-forming cell (CFC) assay.** Total colony-forming cells (CFCs), i.e., granulocyte-macrophage colony-forming units (CFU-GM), erythroid burst-forming units (BFU-E), and multilineage CFU (CFU-GEMM) were assessed in standard methylcellulose cultures. Briefly, 1-ml aliquots of blood ( $5 \times 10^4$  to  $2 \times 10^5$  MNCs) were plated in 35-mm Petri dishes in methylcellulose-based medium (HCC-3434; Stem Cell Technologies) supplemented with recombinant

**EXAMPLE 5****Table 5 — WBC counts in mice treated with rhPIGF and/or rhG-CSF**

| Mobilization Regimen*                          | WBC/ $\mu$ L blood     |                    |
|--|------------------------|--------------------|
|  | Median (range)         | Mean $\pm$ SD      |
| PBS/MSA  | 2,000 (850–4,000)      | 2,165 $\pm$ 929    |
| rhG-CSF (10 $\mu$ g/d)                         | 6,000 (5,200–21,650)   | 9,577 $\pm$ 5,575  |
| rhPIGF (10 $\mu$ g/d)                          | 1,900 (1,050–5,000)    | 2,296 $\pm$ 1,235  |
| rhG-CSF (10 $\mu$ g/d) + rhPIGF (5 $\mu$ g/d)  | 14,400 (11,000–14,600) | 13,333 $\pm$ 2,023 |
| rhG-CSF (10 $\mu$ g/d) + rhPIGF (10 $\mu$ g/d) | 12,800 (5,100–17,350)  | 11,728 $\pm$ 4,968 |

\* BALB/c mice were injected IP for 5 days with either PBS/MSA, rhG-CSF alone (10  $\mu$ g/d), or a combination of rhG-CSF (10  $\mu$ g/d) with rhPIGF (5–10  $\mu$ g/d). Blood samples were collected 2 hours after the last injection of rhPIGF and/or rhG-CSF.

**EXAMPLE 6**

**Table 6 — Frequency of circulating CFCs in mice treated with rhPIGF and/or rhG-CSF**

| Mobilization Regimen*                          | CFCs/ $10^5$ MNCs |               |
|--|-------------------|---------------|
|  | Median (range)    | Mean $\pm$ SD |
| PBS/MSA  | 7 (2–15)          | 8 $\pm$ 3     |
| rhG-CSF (10 $\mu$ g/d)                         | 76 (51–148)       | 82 $\pm$ 29   |
| rhPIGF (10 $\mu$ g/d)                          | 9 (6–21)          | 10 $\pm$ 4    |
| rhG-CSF (10 $\mu$ g/d) + rhPIGF (5 $\mu$ g/d)  | 228 (208–237)     | 224 $\pm$ 14  |
| rhG-CSF (10 $\mu$ g/d) + rhPIGF (10 $\mu$ g/d) | 264 (111–384)     | 256 $\pm$ 77  |

\* BALB/c mice were injected IP for 5 days with either PBS/MSA, rhG-CSF alone (10  $\mu$ g/d), or a combination of rhG-CSF (10  $\mu$ g/d) with rhPIGF (2.5–5  $\mu$ g/d). Blood samples were collected 2 hours after the last injection of rhPIGF and/or rhG-CSF. CFCs include granulocyte-macrophage CFC

(CFU-GM), erythroid burst forming unit (BFU-E), and multipotent CFC (CFU-Mix). CFC data are derived from quadruplicate cultures on samples from each animal.

### EXAMPLE 7

5 Table 7 Absolute number of circulating CFCs in mice treated with rhPIGF and/or rhG-CSF

| Mobilization Regimen*                          | CFCs per ml Blood     |                    |
|--|-----------------------|--------------------|
|  | Median (range)        | Mean $\pm$ SD      |
| PBS/MSA  | 57 (9-288)            | 81 $\pm$ 75        |
| rhG-CSF (10 $\mu$ g/d)                         | 3,129 (1,042-5,518)   | 2,977 $\pm$ 1,126  |
| rhPIGF (10 $\mu$ g/d)                          | 74 (12-236)           | 82 $\pm$ 64        |
| rhG-CSF (10 $\mu$ g/d) + rhPIGF (5 $\mu$ g/d)  | 9,467 (7,514-11,325)  | 9,435 $\pm$ 1,906  |
| rhG-CSF (10 $\mu$ g/d) + rhPIGF (10 $\mu$ g/d) | 11,584 (8,105-17,408) | 12,122 $\pm$ 2,788 |

\* BALB/c mice were injected IP for 5 days with either PBS/MSA, rhG-CSF alone (10  $\mu$ g/d), or a combination of rhG-CSF (10  $\mu$ g/d) with  
 10 rhPIGF (2.5-5  $\mu$ g/d). Blood samples were collected 2 hours after the last injection of rhPIGF and/or rhG-CSF. CFCs include granulocyte macrophage CFC (CFU-GM), erythroid burst forming unit (BFU-E), and multipotent CFC (CFU-Mix). CFC data are derived from quadruplicate cultures on samples from each animal. The absolute number of circulating CFCs in blood is a  
 15 function of the frequency of CFC multiplied by the total number of MNCs per ml blood.

~~EXAMPLE 8~~~~Table 8 Absolute number of circulating LTC ICs in mice treated with rhPIGF and/or rhG-CSF~~

| Mobilization Regimen*                          | LTC-ICs per ml Blood |                 |
|--|----------------------|-----------------|
|  | Median (range)       | Mean $\pm$ SD   |
| PBS/MSA  | 7 (3-29)             | 9 $\pm$ 5       |
| rhG-CSF (10 $\mu$ g/d)                         | 194 (57-337)         | 208 $\pm$ 98    |
| rhPIGF (10 $\mu$ g/d)                          | ND                   | ND              |
| rhG-CSF (10 $\mu$ g/d) + rhPIGF (5 $\mu$ g/d)  | ND                   | ND              |
| rhG-CSF (10 $\mu$ g/d) + rhPIGF (10 $\mu$ g/d) | 1,776 (1,407-1,990)  | 1,724 $\pm$ 294 |

5 ND, not done

~~\* BALB/c mice were injected IP for 5 days with either PBS/MSA, rhG-CSF alone (10  $\mu$ g/d), or a combination of rhG-CSF (10  $\mu$ g/d) with rmPIGF (2.5-5  $\mu$ g/d). Blood samples were collected 2 hours after the last injection of rmPIGF and/or rhG-CSF. The absolute number of circulating LTC IC was assayed in bulk cultures. Test cells ( $5-8 \times 10^6$ ) were seeded into cultures containing a feeder layer of irradiated murine AFT024 cells. After 4 weeks in culture, nonadherent cells and adherent cells harvested by trypsinization were pooled, washed, and assayed together for clonogenic cells. The total number of clonogenic cells (i.e., CFU Mix plus BFU E plus CFU GM) present in 4 week old LTC provides a relative measure of the number of LTC IC originally present in the test suspension. The absolute number of circulating LTC ICs in blood is a function of the frequency of LTC ICs multiplied by the total number of MNCs per ml blood.~~

~~EXAMPLE 9~~

~~Table 9 WBC counts in mice receiving a 12 day treatment with rhPlGF (10 µg/d) and/or rhG-CSF (10 µg/d)~~

| Mobilization Regimen*   | WBC/µL blood   |
|-------------------------|----------------|
|                         | Mean ± SD      |
| PBS/MSA                 | 2,165 ± 929    |
| 5-day rhG-CSF           | 18,683 ± 3,001 |
| 5-day rhG-CSF + rhPlGF  | 16,083 ± 1,227 |
| 8-day rhG-CSF           | 22,017 ± 5,778 |
| 8-day rhG-CSF + rhPlGF  | 16,000 ± 6,354 |
| 10-day rhG-CSF          | 21,500 ± 3,317 |
| 10-day rhG-CSF + rhPlGF | 24,800 ± 6,699 |
| 12-day rhG-CSF          | 43,100 ± 8,598 |
| 12-day rhG-CSF + rhPlGF | 46,167 ± 5,678 |

- 5      \* BALB/c mice were injected IP for 12 days with either PBS/MSA, rhG-CSF alone (10 µg/d), or a combination of rhG-CSF (10 µg/d) with rhPlGF (10 µg/d). Blood samples were collected after 5, 8, 10, and 12 days of treatment.

~~EXAMPLE 10~~

~~Table 10 — Frequency of circulating CFCs in mice receiving a 12-day treatment with rhPlGF (10 µg/d) and/or rhG-CSF (10 µg/d)~~

| Mobilization Regimen*   | CFCs/10 <sup>5</sup> MNCs |
|-------------------------|---------------------------|
|                         | Mean ± SD                 |
| PBS/MSA                 | 8 ± 3                     |
| 5-day rhG-CSF           | 63 ± 12                   |
| 5-day rhG-CSF + rhPlGF  | 297 ± 80                  |
| 8-day rhG-CSF           | 70 ± 5                    |
| 8-day rhG-CSF + rhPlGF  | 180 ± 20                  |
| 10-day rhG-CSF          | 102 ± 8                   |
| 10-day rhG-CSF + rhPlGF | 274 ± 34                  |
| 12-day rhG-CSF          | 106 ± 19                  |
| 12-day rhG-CSF + rhPlGF | 299 ± 49                  |

- 5       \* BALB/c mice were injected IP for 12 days with either PBS/MSA, rhG-CSF alone (10 µg/d), or a combination of rhG-CSF (10 µg/d) with rhPlGF (10 µg/d). Blood samples were collected after 5, 8, 10, and 12 days of treatment. CFCs include granulocyte macrophage CFC (CFU-GM), erythroid burst forming unit (BFU-E), and multipotent CFC (CFU-Mix). CFC data are
- 10      derived from quadruplicate cultures on samples from each animal.

~~EXAMPLE 11~~~~Table 11—Absolute number of circulating CFCs in mice receiving a 12-day treatment with rhPIGF (10 µg/d) and/or rhG-CSF (10 µg/d)~~

| Mobilization Regimen*   | CFCs per ml Blood |
|-------------------------|-------------------|
|                         | Mean ± SD         |
| PBS/MSA                 | 81 ± 75           |
| 5-day rhG-CSF           | 3,427 ± 232       |
| 5-day rhG-CSF + rhPIGF  | 11,649 ± 1,827    |
| 8-day rhG-CSF           | 6,361 ± 1,931     |
| 8-day rhG-CSF + rhPIGF  | 10,341 ± 799      |
| 10-day rhG-CSF          | 4,335 ± 923       |
| 10-day rhG-CSF + rhPIGF | 14,104 ± 2,687    |
| 12-day rhG-CSF          | 10,968 ± 2,183    |
| 12-day rhG-CSF + rhPIGF | 32,024 ± 4,915    |

- 5       \* BALB/c mice were injected IP for 12 days with either PBS/MSA, rhG-CSF alone (10 µg/d), or a combination of rhG-CSF (10 µg/d) with rhPIGF (10 µg/d). Blood samples were collected after 5, 8, 10, and 12 days of treatment. CFCs include granulocyte-macrophage CFC (CFU-GM), erythroid burst-forming unit (BFU-E), and multipotent CFC (CFU-Mix). CFC data are
- 10      derived from quadruplicate cultures on samples from each animal. The absolute number of circulating CFCs in blood is a function of the frequency of CFC multiplied by the total number of MNCs per ml blood.

**EXAMPLES 12-18— 5-11 - mobilizing effects of PIGF/G-CSF combination in a non-human primate model**

15      **MATERIALS AND METHODS**

Experimental design. A cohort of Rhesus Monkeys (n = 4) was initially mobilized with G-CSF alone (100 µg/kg/day, SC, for 5 days) (cycle 1), and after a 6-week wash-out period, received a second mobilization

( $\geq 1$  colony) or negative (no colony) and the LTC-IC frequencies were calculated by using L-Calc software (Stem Cell Technologies). The absolute numbers of circulating LTC-IC were assessed in bulk cultures (46). Briefly, test cells ( $5 - 8 \times 10^6$ ) were resuspended in complete medium and seeded into  
5 cultures containing a feeder layer of irradiated murine M2-10B4 cells ( $3 \times 10^4/\text{cm}^2$ ). After 5 weeks in culture, nonadherent cells and adherent cells harvested by trypsinization were pooled, washed, and assayed together for clonogenic cells. The total number of clonogenic cells (i.e., CFU-GEMM plus BFU-E plus CFU-GM) present in 5-week-old LTC provides a relative measure  
10 of the number of LTC-IC originally present in the test suspension. Absolute LTC-IC values were calculated by dividing the total number of clonogenic cells by 4, which is the average output of clonogenic cells per LTC-IC.

#### EXAMPLE 12.5

--- **Circulating WBCs.** A 5-day administration of rhG-CSF alone induced  
15 an average 5-fold increment in the mean ( $\pm$ SD) numbers of WBCs, as compared to pretreatment values. Addition of 130 or 260  $\mu\text{g/kg}$  rhPIGF to rhG-CSF resulted in a modest increase of WBC values detected on day 5 of treatment.

Table 12-5 - WBC counts in Rhesus monkeys treated with rhG-CSF alone or rhPlGF plus rhG-CSF

|     | WBC counts per $\mu\text{L}$ blood *                      |   |   |
|-----|---|---|---|
|     | Cycle 1   | Cycle 2   | Cycle 3   |
| Day | rhG-CSF<br>(100 $\mu\text{g/kg/day}$ , SC, for<br>5 days) | rhPlGF<br>(130 $\mu\text{g/kg}$ , IV, for 5<br>days)<br>+ rhG-CSF<br>(100 $\mu\text{g/kg/day}$ , SC, for<br>5 days) | rhPlGF<br>(260 $\mu\text{g/kg}$ , IV, for 5<br>days)<br>+ rhG-CSF<br>(100 $\mu\text{g/kg/day}$ , SC, for<br>5 days) |
| 1   | 8,708 $\pm$ 2,458   | 13,498 $\pm$ 5,514  | 8,370 $\pm$ 1,585   |
| 2   | 31,313 $\pm$ 3,889  | 24,533 $\pm$ 2,789  | 41,180 $\pm$ 7,364  |
| 3   | 40,600 $\pm$ 6,274  | 35,388 $\pm$ 2,207  | 44,085 $\pm$ 6,588  |
| 4   | 43,055 $\pm$ 6,562  | 39,440 $\pm$ 6,744  | 37,960 $\pm$ 3,598  |
| 5   | 43,523 $\pm$ 13,790                                       | 60,040 $\pm$ 9,508  | 49,048 $\pm$ 7,120  |
| 8   | 14,363 $\pm$ 4,163  | 23,073 $\pm$ 9,017  | 17,783 $\pm$ 5,964  |
| 10  | 12,145 $\pm$ 5,421  | 16,398 $\pm$ 8,314  | 11,150 $\pm$ 2,915  |

\* Rhesus monkeys ( $n = 4$ ) received three mobilization cycles separated by a 6-week washout period. Mobilization was elicited at cycle 1 by rhG-CSF alone (100  $\mu\text{g/kg/day}$ , SC, day 1 - 5), at cycle 2 by a combination of rhPlGF (130  $\mu\text{g/kg}$ , IV, day 1 - 5) plus rhG-CSF (100  $\mu\text{g/kg/day}$ , SC, day 1 - 5), and at cycle 3 by a combination of rhPlGF (260  $\mu\text{g/kg}$ , IV, day 1 - 5) plus rhG-CSF (100  $\mu\text{g/kg/day}$ , SC, day 1 - 5). WBC counts were analyzed daily during treatment (days 1 to 5), as well as 3 and 5 days post-cessation of therapy. Data are expressed as mean  $\pm$  SD.

#### EXAMPLE 13.6

**Frequency of CFCs.** As compared to baseline values, the mean frequencies of blood CFCs (per  $10^5$  MNCs) detected at peak were increased by 19-, 53-, and 52-fold under rhG-CSF alone, rhG-CSF/rhPlGF (130  $\mu\text{g/kg}$ ), and

rhG-CSF/rhPlGF (260 µg/kg), respectively. As compared to rhG-CSF alone, the combined rhPlGF/rhG-CSF treatment induced a 2-fold increase of CFC frequency on the day of peak.

Table 13- 6 - Frequency of circulating CFCs in Rhesus monkeys treated with rhG-CSF alone or rhPlGF plus rhG-CSF

|     | CFCs/10 <sup>5</sup> MNCs *                   |   |   |
|-----|---|---|---|
|     | Cycle 1                                       | Cycle 2   | Cycle 3   |
| Day | rhG-CSF<br>(100 µg/kg/day, SC,<br>for 5 days) | rhPlGF<br>(130 µg/kg, IV, for 5<br>days)<br>+ rhG-CSF<br>(100 µg/kg/day, SC,<br>for 5 days) | rhPlGF<br>(260 µg/kg, IV, for 5<br>days)<br>+ rhG-CSF<br>(100 µg/kg/day, SC,<br>for 5 days) |
| 1   | 6 ± 1   | 4 ± 1   | 5 ± 3   |
| 2   | 4 ± 2   | 9 ± 1   | 19 ± 8  |
| 3   | 9 ± 1   | 39 ± 13   | 48 ± 26   |
| 4   | 114 ± 51                                      | 213 ± 87  | 245 ± 151   |
| 5   | 63 ± 26                                       | 196 ± 26  | 261 ± 83  |
| 8   | 66 ± 11                                       | 40 ± 11   | 60 ± 39   |
| 10  | 10 ± 7  | 19 ± 10   | 21 ± 18   |

\* Rhesus monkeys (n = 4) received three mobilization cycles separated by a 6-week washout period. Mobilization was elicited at cycle 1 by rhG-CSF alone (100 µg/kg/day, SC, day 1 - 5), at cycle 2 by a combination of rhPlGF (130 µg/kg, IV, day 1 - 5) plus rhG-CSF (100 µg/kg/day, SC, day 1 - 5), and at cycle 3 by a combination of rhPlGF (260 µg/kg, IV, day 1 - 5) plus rhG-CSF (100 µg/kg/day, SC, day 1 - 5). CFCs were analyzed daily during treatment (days 1 to 5), as well as 3 and 5 days post-cessation of therapy. Data are expressed as mean ± SD. CFCs include granulocyte-macrophage CFC (CFU-GM), erythroid burst-forming unit (BFU-E), and multipotent CFC

(CFU-Mix). CFC data are derived from quadruplicate cultures on samples from each animal.

#### **EXAMPLE 14 7**

**Absolute values of CFCs.** Absolute numbers of circulating CFCs in blood were calculated as a function of the frequency of CFCs multiplied by the total number of MNCs per ml blood. As compared to baseline values, treatment with rhG-CSF alone, rhG-CSF/rhPlGF (130 µg/kg), and rhG-CSF/rhPlGF (260 µg/kg) resulted in a 85- 335- and 358-fold increase of CFCs, respectively. At cycles 2 and 3, the peak levels of CFCs were increased by 4- and 5-fold over cycle 1 (rhG-CSF alone).

**Table 14- 7 - Absolute numbers of circulating CFCs in Rhesus Monkeys treated with rhG-CSF alone or rhPlGF plus rhG-CSF**

|            | <b>CFCs per ml blood *</b>                    |   |   |
|------------|---|---|---|
|            | <b>Cycle 1</b>                                | <b>Cycle 2</b>  | <b>Cycle 3</b>  |
| <b>Day</b> | <b>rhG-CSF(100 µg/kg/day, SC, for 5 days)</b> | <b>rhPlGF (130 µg/kg, IV, for 5 days)<br/>+ rhG-CSF (100 µg/kg/day, SC, for 5 days)</b> | <b>rhPlGF (260 µg/kg, IV, for 5 days)<br/>+ rhG-CSF (100 µg/kg/day, SC, for 5 days)</b> |
| <b>1</b>   | 134 ± 9                                       | 138 ± 38  | 170 ± 129   |
| <b>2</b>   | 344 ± 207                                     | 724 ± 254   | 6,552 ± 4,365   |
| <b>3</b>   | 472 ± 60                                      | 6,420 ± 4,775   | 9,634 ± 7,006   |
| <b>4</b>   | 11,406 ± 4,093                                | 32,347 ± 14,206   | 53,002 ± 25,250   |
| <b>5</b>   | 5,397 ± 3,074                                 | 46,283 ± 8,287  | 60,777 ± 8,563  |
| <b>8</b>   | 3,952 ± 2,666                                 | 4,532 ± 3,714   | 3,719 ± 1,899   |
| <b>10</b>  | 224 ± 164                                     | 448 ± 168   | 943 ± 994   |

\* Rhesus monkeys (n = 4) received three mobilization cycles separated by a 6-week washout period. Mobilization was elicited at cycle 1 by rhG-CSF

alone (100 µg/kg/day, SC, day 1 - 5), at cycle 2 by a combination of rhPlGF (130 µg/kg, IV, day 1 - 5) plus rhG-CSF (100 µg/kg/day, SC, day 1 - 5), and at cycle 3 by a combination of rhPlGF (260 µg/kg, IV, day 1 - 5) plus rhG-CSF (100 µg/kg/day, SC, day 1 - 5). CFCs were analyzed daily during treatment (days 1 to 5), as well as 3 and 5 days post-cessation of therapy. Data are expressed as mean ± SD. CFCs include granulocyte-macrophage CFC (CFU-GM), erythroid burst-forming unit (BFU-E), and multipotent CFC (CFU-Mix). CFC data are derived from quadruplicate cultures on samples from each animal. The absolute number of circulating CFCs in blood is a function of the frequency of CFC multiplied by the total number of MNCs per ml blood.

#### EXAMPLE 15 8

**Frequency of HPP-CFCs.** As compared to baseline values, the mean frequencies of blood HPP-CFCs (per  $10^5$  MNCs) detected on day 5 of mobilization were increased by 5-, and 12-fold under rhG-CSF alone or rhG-CSF/rhPlGF (130 µg/kg), respectively. As compared to rhG-CSF alone, the combined rhPlGF/rhG-CSF treatment induced a 2-fold increase of HPP-CFC frequency on the day of peak.

**Table 15- 8 --Frequency of circulating HPP-CFCs in Rhesus monkeys treated with rhG-CSF alone or rhPIGF plus rhG-CSF**

|     | HPP-CFCs/10 <sup>5</sup> MNCs *            |   |
|-----|--|---|
|     | Cycle 1                                    | Cycle 2   |
| Day | rhG-CSF<br>(100 µg/kg/day, SC, for 5 days) | rhPIGF<br>(130 µg/kg, IV, for 5 days)<br>+ rhG-CSF<br>(100 µg/kg/day, SC, for 5 days) |
| 1   | 4 ± 1                                      | 3 ± 1   |
| 2   | 6 ± 1                                      | 3 ± 1   |
| 3   | 13 ± 4                                     | 11 ± 3  |
| 4   | 15 ± 4                                     | 27 ± 10   |
| 5   | 20 ± 9                                     | 37 ± 8  |
| 8   | 18 ± 6                                     | 6 ± 4   |
| 10  | 6 ± 1                                      | 5 ± 4   |

\* Rhesus monkeys (n = 4) received three mobilization cycles separated by a 6-week washout period. Mobilization was elicited at cycle 1 by rhG-CSF alone (100 µg/kg/day, SC, day 1 - 5), at cycle 2 by a combination of rhPIGF (130 µg/kg, IV, day 1 - 5) plus rhG-CSF (100 µg/kg/day, SC, day 1 - 5), and at cycle 3 by a combination of rhPIGF (260 µg/kg, IV, day 1 - 5) plus rhG-CSF (100 µg/kg/day, SC, day 1 - 5). HPP-CFCs were analyzed daily during treatment (days 1 to 5), as well as 3 and 5 days post-cessation of therapy. Data are expressed as mean ± SD. HPP-CFC data are derived from quadruplicate cultures on samples from each animal.

#### **EXAMPLE 16 9**

**Absolute values of HPP-CFCs.** The absolute number of HPP-CFCs per ml blood detected on day 5 of rhG-CSF therapy was 17-fold higher than pre-treatment values. Monkeys receiving the combined rhG-CSF/rhPIGF (130

$\mu\text{g/kg}$ )-treatment showed a 158-fold increase of HPP-CFCs as compared to baseline values. At cycle 2, the level of day-5 HPP-CFCs was increased by 5-fold over cycle 1.

Table 16- 9 - Absolute numbers of circulating HPP-CFC in Rhesus

5 Monkeys treated with rhG-CSF alone or rhPlGF plus rhG-CSF

|     | HPP-CFCs per ml blood *                                |   |
|-----|--|---|
|     | Cycle 1  | Cycle 2   |
| Day | rhG-CSF<br>(100 $\mu\text{g/kg/day}$ , SC, for 5 days) | rhPlGF<br>(130 $\mu\text{g/kg}$ , IV, for 5 days)<br>+ rhG-CSF<br>(100 $\mu\text{g/kg/day}$ , SC, for 5 days) |
| 1   | 96 $\pm$ 17  | 54 $\pm$ 49   |
| 2   | 493 $\pm$ 218  | 258 $\pm$ 34  |
| 3   | 683 $\pm$ 155  | 1,709 $\pm$ 989   |
| 4   | 1,521 $\pm$ 332  | 3,883 $\pm$ 1,309   |
| 5   | 1,593 $\pm$ 405  | 8,557 $\pm$ 1,142   |
| 8   | 998 $\pm$ 541  | 603 $\pm$ 384   |
| 10  | 121 $\pm$ 52   | 121 $\pm$ 87  |

\* Rhesus monkeys (n = 4) received three mobilization cycles separated by a 6-week washout period. Mobilization was elicited at cycle 1 by rhG-CSF alone (100  $\mu\text{g/kg/day}$ , SC, day 1 - 5), at cycle 2 by a combination of rhPlGF (130  $\mu\text{g/kg}$ , IV, day 1 - 5) plus rhG-CSF (100  $\mu\text{g/kg/day}$ , SC, day 1 - 5), and at cycle 3 by a combination of rhPlGF (260  $\mu\text{g/kg}$ , IV, day 1 - 5) plus rhG-CSF (100  $\mu\text{g/kg/day}$ , SC, day 1 - 5). HPP-CFC counts were analyzed daily during treatment (days 1 to 5), as well as 3 and 5 days post-cessation of therapy. Data are expressed as mean  $\pm$  SD. HPP-CFCs data are derived from quadruplicate cultures on samples from each animal. The absolute number of circulating HPP-CFCs in blood is a function of the frequency of HPP-CFCs multiplied by

the total number of MNCs per ml blood.

### EXAMPLE 17 10

Frequency of LTC-ICs. Analysis of the LTC-IC frequency by a limiting dilution assay showed that the combined administration of rhPlGF (130 µg/kg) and rhG-CSF resulted in an average increase the LTC-IC frequency by 11-fold (1 in 5,829 vs 1 in 64,064 cells), as compared to rhG-CSF alone.

Table 17-10 - Frequency of circulating LTC-ICs in Rhesus Monkeys receiving a 5-day course of rhG-CSF alone or rhPlGF plus rhG-CSF

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| Animal No. | Mobilization Regimen         | LTC-IC Frequency (mean)* | 95% CI          |                 | LTC-IC per 10 <sup>5</sup> MNCs |
|------------|------------------------------|--------------------------|-----------------|-----------------|---------------------------------|
|            |                              |                          | Lower Frequency | Upper Frequency |                                 |
| 1          | rhG-CSF                      | 1/84,265                 | 1/69,209        | 1/102,598       | 1.2                             |
| 2          | rhG-CSF                      | 1/65,835                 | 1/54,341        | 1/79,761        | 1.5                             |
| 3          | rhG-CSF                      | ne **                    | ne              | ne              | ne                              |
| 4          | rhG-CSF                      | 1/42,091                 | 1/34,837        | 1/50,854        | 2.4                             |
| 1          | rhPlGF (130 µg/kg) + rhG-CSF | 1/4,009                  | 1/5,977         | 1/2,689         | 24.9                            |
| 2          | rhPlGF (130 µg/kg) + rhG-CSF | 1/7,562                  | 1/11,100        | 1/5,152         | 13.2                            |
| 3          | rhPlGF (130 µg/kg) + rhG-CSF | ne                       | ne              | ne              | ne                              |
| 4          | rhPlGF (130 µg/kg) + rhG-CSF | 1/5,916                  | 1/8,725         | 1/4,011         | 16.9                            |

\* The frequency of LTC-IC was assayed under limiting dilution conditions using the murine M2-10B4 cell line as stromal layer. Blood samples were collected on day 5 of mobilization therapy. Serial dilutions of test cells ( $2 \times 10^5$  to  $3 \times 10^3$ ) were cultured for 5 weeks and 16 to 22 replicates were plated for each test cell dose. After 5 weeks, nonadherent and adherent

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cells from individual wells were assayed for clonogenic cells and the LTC-IC frequencies were calculated using Poisson statistics and the method of maximum likelihood.

### EXAMPLE 18 11

- 5        **Absolute values of LTC-ICs.** Under rhG-CSF alone, absolute numbers of circulating LTC-ICs were increased by 53-fold on day 4 of treatment as compared to baseline values. The combined rhG-CSF/rhPIGF (130 µg/kg) treatment increased LTC-ICs by 389-fold as compared to pretreatment values, and by 15-fold as compared to rhG-CSF alone.

- 10        Table 18- 11 - Absolute numbers of circulating LTC-ICs in Rhesus Monkeys treated with rhG-CSF alone or rhPIGF plus rhG-CSF

|     | LTC-ICs per ml blood *                     |   |
|-----|--|---|
|     | Cycle 1                                    | Cycle 2   |
| Day | rhG-CSF<br>(100 µg/kg/day, SC, for 5 days) | rhPIGF<br>(130 µg/kg, IV, for 5 days)<br>+ rhG-CSF<br>(100 µg/kg/day, SC, for 5 days) |
| 1   | 4 ± 7                                      | 8 ± 5   |
| 2   | 92 ± 43                                    | 56 ± 20   |
| 3   | 111 ± 30                                   | 624 ± 340   |
| 4   | 211 ± 41                                   | 742 ± 176   |
| 5   | 130 ± 25                                   | 3,115 ± 988   |
| 8   | 63 ± 22                                    | 533 ± 270   |
| 10  | 6 ± 2                                      | 112 ± 40  |

- \* Rhesus monkeys (n = 4) received three mobilization cycles separated by a 6-week washout period. Mobilization was elicited at cycle 1 by rhG-CSF alone (100 µg/kg/day, SC, day 1 - 5), at cycle 2 by a combination of rhPIGF (130 µg/kg, IV, day 1 - 5) plus rhG-CSF (100 µg/kg/day, SC, day 1 - 5), and at
- 15